Effects of Bovine Somatotropin on Uterine Genes Related to the Prostaglandin Cascade in Lactating Dairy Cows*

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ABSTRACT

Multiparous Holstein cows, averaging 80 d in milk, were used to examine the effect of exogenous bovine somatotropin (bST) on uterine expression of estrogen receptor α (ER α), prostaglandin endoperoxide synthase-2 (PGHS-2), and peroxisome proliferator-activated receptor δ (PPAR δ). About 12 h before expected ovulation in a synchronization protocol, cows were assigned to receive bST (500 mg, $n = 11$) or serve as untreated controls ($n = 10$). Cows that ovulated ($n = 9$ bST, 8 control) were divided within treatment to be killed on d 3 or 7 postovulation. Samples of intercaruncular endometrial tissue from uterine horns ipsilateral to the corpus luteum were collected and stored at -80°C for subsequent mRNA analyses. Endometrial concentrations of ER α and PGHS-2 mRNA transcripts were greater on d 7 than on d 3 of the estrous cycle, but did not differ between treatments. Compared with untreated cows, short-term bST treatment decreased PGHS-2 protein expression at d 7 of the estrous cycle. Concentration of PPAR δ mRNA transcript in the uterus decreased between d 3 and 7 of the estrous cycle and was negatively correlated with ER α and PGHS-2 mRNA concentrations. Short-term administration of bST to lactating dairy cows had minimal effects on uterine genes encoding ER α , PGHS-2, and PPAR δ at d 3 and 7 of the estrous cycle but there may be an inverse relationship between PPAR δ and uterine expression of ER α and PGHS-2 genes.

(**Key words:** uterus, gene, bovine somatotropin, receptor)

Abbreviation key: ER α = estrogen receptor alpha, PGHS = prostaglandin endoperoxide synthase, PPAR = peroxisome proliferator-activated receptor, TBS = Tris-buffered saline.

INTRODUCTION

Episodic release of PGF $_{2\alpha}$ from the uterus is responsible for luteolytic signal during the estrous cycle in domestic ruminants (McCracken et al., 1972; Thatcher et al., 1984). It has been proposed that estrogens from ovarian follicles interact with estrogen receptor alpha (ER α) to increase oxytocin receptor gene expression, which ultimately leads to pulsatile release of PGF $_{2\alpha}$ (Beard and Lamming, 1994; Kim et al., 2003). The rate-limiting step in eicosanoid synthesis is the cleavage of sn-2 fatty acyl ester bond of membrane phospholipids by cytosolic phospholipase A $_2$ (Van den Bosch, 1980; Irvine, 1982). The arachidonic acid that is released by phospholipid hydrolysis is acted on by prostaglandin endoperoxide synthase (PGHS) to form PGH-2, which then is converted to PGF by a reductase. There are 2 forms of PGHS that have been characterized, a constitutively expressed PGHS-1, and an induced PGHS-2 (Smith et al., 1996). In cattle, the synthesis and activity of PGHS-2 must be attenuated for pregnancy to be maintained (Thatcher et al., 1997). Whether and how exogenous bST interacts with the PGF cascade in cattle has not been fully elucidated.

Peroxisome proliferator-activated receptors (PPAR) have been studied traditionally for their roles in lipid metabolism and metabolic diseases (Chinetti et al., 2000). There are 3 subtypes of PPAR (PPAR α , δ/β , and γ), all of which have distinct patterns of expression and functional roles (Braissant and Wahli, 1998). Subtype PPAR α is mainly expressed in tissues in which fatty acid catabolism is significant, such as the liver, heart, and skeletal muscle (Jalouli et al., 2003; Lee et al., 2003; Schiffrin et al., 2003). Subtype PPAR γ is highly expressed in adipose tissue, where it regulates adipocyte differentiation, lipid storage, and insulin sensitivity (Chawla et al., 2003; Schiffrin et al., 2003). Much less is known about the function of PPAR δ , although it is highly expressed in the brain, colon, and skin (Braissant and Wahli, 1998; Matsuura et al., 1999; Mano et al., 2000). In mice, PPAR δ deficiency leads to placental defects and results in frequent midgestational lethality (Barak et al., 2002), suggesting that this nuclear recep-

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tor may play an important role in the control of reproductive processes in mammalian species.

Administration of recombinant bST to dairy cows has become a common management practice in the United States for enhancing milk production (Peel and Bauman, 1987). However, this practice has raised numerous concerns, as exogenous bST initially was shown to decrease fertility in dairy cattle (Cole et al., 1992; Zhao et al., 1992). It was suggested that the negative effect of supplemental bST on fertility might be caused, in part, by lower estrous detection rates (Kirby et al., 1997; Lefebvre and Block, 1992). Recent studies indicated that exogenous bST increased pregnancy rates in lactating dairy cows when administered at estrus in repeat breeding cows (Morales-Roura et al., 2001) or when combined with a regimen for synchronization of ovulation and timed AI (Moreira et al., 2000, 2001). This raises the possibility that bST administration to dairy cows during the peri-ovulatory period may have a positive effect on the endocrine and biochemical signals between the conceptus and maternal uterus at the time of pregnancy establishment.

Given the lack of information relating uterine components of the PGF cascade and supplemental bST, the objective of this study was to examine the effect of exogenous bST on uterine endometrial expression of ER α , PGHS-2, and PPAR δ in lactating Holstein cows, and to determine whether those responses vary with stage of the estrous cycle.

MATERIALS AND METHODS

Experimental Design

Twenty-one multiparous Holstein cows (average DIM = 80) were used in a completely randomized design to examine the effect of exogenous bST on uterine endometrial expression of ER α , PGHS-2, and PPAR δ during the estrous cycle. Detailed cow management and experimental manipulations were described previously (Pershing et al., 2002). Briefly, lactating Holstein cows were milked 3 times daily and fed a TMR that contained 1.65 Mcal/kg and 18.1% CP (DM basis) during the entire experiment (March to May 2000). At approximately 44 DIM, cows were presynchronized with an injection of GnRH (Cystorelin, Merial Ltd., Iselin, NJ; 100 mg, i.m.) followed 7 d later with PGF $_{2\alpha}$ (Lutalyse, Pharmacia Animal Health, Kalamazoo, MI; 25 mg, i.m.). Twelve days after the PGF $_{2\alpha}$ injection, the Ovsynch program was initiated with an i.m. injection of GnRH (100 mg) followed 7 d later with PGF $_{2\alpha}$ (25 mg). Forty-eight hours after the administration of PGF $_{2\alpha}$, cows received a second dose of GnRH to induce ovulation. On the day of expected ovulation (approximately 16 h after the second GnRH administration), cows were assigned randomly

to serve as untreated controls (n = 10) or to receive an i.m. injection of bST (Posilac, Monsanto Co., St. Louis, MO; 500 mg). Within each treatment, cows that ovulated (n = 9 bST, 8 control) were killed on either d 3 or 7 following initiation of bST treatment. Ovulation was verified by ultrasonography within 48 h of the second GnRH dose and later confirmed at slaughter.

RNA Isolation and Analysis

Reproductive tracts were collected and brought to the laboratory within 15 min of slaughter. Uterine horns ipsilateral to the corpus luteum were trimmed free of the broad ligament, and samples (~5 g) of intercaruncular endometrial tissue were collected and immediately frozen at -80°C for subsequent RNA and Western blot analyses. Total cellular RNA was isolated from endometrial tissues using TriZol reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions. Samples of RNA (20 μ g/lane) were fractionated in a 1% (wt/vol) agarose-formaldehyde gel, blotted to Biotrans nylon membranes (ICN, Irvine, CA), and prehybridized in Rapid-Hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ) at 60°C for 1 h. The filters then were hybridized for 2 h at 60°C in Rapid-Hyb buffer with 32 P-labeled ER α (Ing et al., 1996), bovine PGHS-2 (a gift from Jean Sirois, Universite de Montreal, St-Hyacinthe, Canada), and bovine PPAR δ (amplified by reverse transcription-PCR from bovine endometrial RNA) cDNA probes. The cDNA probe for PPAR δ was generated using a set of primers (forward: 5'-CACTCTCACTGCTGGACAA-3'; reverse: 5'-TGCG GTTCTTCTTCTGGATT-3') designed from the bovine PPAR δ sequence (GenBank accession number: AF229357). The size (216 bp) and identity of the PCR product were further verified by DNA sequencing before its use in Northern blot analyses. The membranes were washed sequentially in 2 $\times$ saline sodium citrate/0.1% (wt/vol) SDS at room temperature for 20 min and 0.1 $\times$ saline sodium citrate/0.1% (wt/vol) SDS at 50°C for 15 min. Hybridization signals were detected by exposing membranes to x-ray film (X-Omat Blue XB 1, Eastman Kodak Co., Rochester, NY) at -80°C for 24 to 48 h. Hybridization signals for each target gene were quantified by densitometric scanning (Kodak Electrophoresis Documentation and Analysis System 290, Eastman Kodak Co.). Following autoradiography, the membranes were stripped with 1% (wt/vol) SDS and rehybridized with 18S ribosomal RNA probe to verify the consistency of RNA loading and specificity of treatment effect.

Western Blot Analysis

The abundance of ER α , PGHS-2, and PPAR δ in uterine endometrium was examined by Western blot analy-

Figure 1. Effects of bST on uterine endometrial expression of estrogen receptor α (ER α) mRNA in control (d 3, lanes 1 to 3; d 7, lanes 1 to 5) and bST-treated (d 3, lanes 4 to 7; d 7, lanes 6 to 10) cows. Each lane represents a different cow. Day of estrous cycle differed, $P = 0.02$.

sis. Total protein was extracted from intercaruncular endometrial tissues (300 to 400 mg) by tissue homogenization in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM EDTA, 2 mM ethylene glycol-bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 50 mM NaF, 1% (wt/vol) Nonidet P-40, 20 mM b-glycerophosphate, 2 mM Na_3VO_4 , 10% (wt/vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 g/mL each of aprotinin, leupeptin, and pepstatin; Binelli et al., 2000]. Protein concentrations in cell lysates were determined by the Bradford method (Bradford, 1976),

with BSA used as the standard. For each endometrial sample, 100 μg of protein was subjected to 7.5% (wt/vol) SDS-PAGE in the presence of β -mercaptoethanol followed by electrotransfer onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) at 4°C overnight. Following electrotransfer, the membranes were transiently stained with Ponceau S solution (Sigma Chemical Co., St. Louis MO) to determine the efficiency of protein transfer. The membranes were blocked in Tris-buffered saline (TBS) containing 5% (wt/vol) Carnation instant nonfat dry milk for 2 h at

Figure 2. Effects of bST on uterine endometrial expression of prostaglandin endoperoxide synthase-2 (PGHS-2) mRNA in control (d 3, lanes 1 to 3; d 7, lanes 1 to 5) and bST-treated (d 3, lanes 4 to 7; d 7, lanes 6 to 10) cows. Each lane represents a different cow. Day of estrous cycle differed, $P < 0.01$.

room temperature. The membranes were then incubated with 200 $\mu\text{g/mL}$ of primary antibodies against $\text{ER}\alpha$ (mouse monoclonal anti- $\text{ER}\alpha$, Amersham), PGHS-2 (rabbit polyclonal anti-PGHS-2, Cayman Chemical Co., Ann Arbor, MI), or $\text{PPAR}\delta$ (rabbit polyclonal anti- $\text{PPAR}\delta$, Santa Cruz Biotechnology Inc., Santa Cruz, CA) in a total volume of 10 mL of TBS for 2 h at room temperature. After incubation, the membranes were washed with 3 changes of 0.1% (wt/vol) Tween-20 TBS for 20 min. The membranes were then incubated with secondary antibodies (antimouse horseradish peroxidase for $\text{ER}\alpha$, Amersham; antirabbit horseradish peroxidase for PGHS-2 and $\text{PPAR}\delta$, Cayman), washed with TBS, and blotted dry. The protein bands were visualized by enhanced chemiluminescence following the manufacturer's instructions (Renaissance Western Blot

Chemiluminescence Reagent Plus, NEN Life Science Products, Inc., Boston, MA). The relative abundance of each protein was detected by exposing immunoblots to x-ray film (Super RX, Fuji Photo Film Co., Tokyo, Japan) for 10 to 30 s. Protein signals were quantified by densitometric analysis (Kodak EDAS 290).

Statistical Analyses

Messenger RNA and protein responses were evaluated by least squares ANOVA using the GLM procedure of the SAS software package (SAS Inst., Inc., Cary, NC). The mathematical model for uterine mRNA and protein responses included treatment, day of estrous cycle, and treatment $\times$ day interaction. For all target genes, the densitometric values were expressed as ratios of target

Figure 3. Effects of bST on uterine endometrial expression of peroxisome proliferator-activated receptor δ (PPAR δ) mRNA in control (d 3, lanes 1 to 3; d 7, lanes 1 to 4) and bST-treated (d 3, lanes 4 to 7; d 7, lanes 5 to 9) cows. Each lane represents a different cow. Day of estrous cycle differed, $P < 0.01$.

Table 1. Pearson (upper right) and partial¹ (lower left) correlation coefficients for uterine endometrial estrogen receptor α (ER α), prostaglandin endoperoxide synthase-2 (PGHS-2), and peroxisome proliferator-activated receptor δ (PPAR δ) mRNA transcripts.

	ER α	PGHS-2	PPAR δ
ER α	—	0.84**	-0.52**
PGHS-2	0.87**	—	-0.81**
PPAR δ	0.07	0.19	—

¹Partial correlations were adjusted for treatment, day of estrous cycle, and treatment $\times$ day interaction. **Significant correlation at $P < 0.01$.

gene values over the corresponding 18S rRNA values. Pearson and partial correlations among endometrial genes and proteins were determined using the CORR and MANOVA procedures, respectively. Partial correlations were adjusted for treatment, day of estrous cycle, and treatment $\times$ day interaction.

RESULTS

Uterine ER α , PGHS-2, and PPAR δ Genes

Uterine endometrial mRNA encoding ER α , PGHS-2, and PPAR δ were readily detectable on d 3 and 7 of the

When averaged within treatments and stages of the estrous cycle, the concentration of the full length ER α protein (67 kDa) was unaffected by bST or day of the

DISCUSSION

The observation that bST receptors are expressed in several reproductive tissues (Kirby et al., 1996) raises the possibility that bST may play a direct role in the control of mammalian reproductive function. In the

thesis may vary depending on the physiological state of the experimental animal (i.e., lactation, stage of estrous cycle, and pregnancy).

Uterine ER α is known to be up regulated by estradiol (Wu et al., 1996; Ing and Tornesi, 1997; Ing and Ott, 1999) and is generally maximally expressed at estrus in the bovine uterus (Meikle et al., 2001). In the cow, peripheral estradiol exhibits 1 or 2 secondary peaks coinciding with the emergence of 1 or 2 nonovulatory dominant follicles during the interestrus interval (Savio et al., 1988; Sirois and Fortune, 1988; Badinga et al., 1992). It is conceivable that the greater ER α and PGHS-2 mRNA concentrations detected in the bovine endometrium at d 7 of the estrous cycle may reflect the subtle rise in plasma E $_2$ that originates from the dominant follicle of the first follicular wave (Badinga et al., 1992). Alternatively, the previously documented inhibitory effects of progesterone on expression of ER α mRNA (Spencer et al., 1996; Wu et al., 1996; McDowell et al., 1999) may occur at later stages of the luteal phase that were not examined in the current study.

Peroxisome proliferator-activated receptors are ligand-activated transcription factors that regulate multiple physiological processes, including reproduction, development, and energy metabolism (Chinetti et al., 2000; Panadero et al., 2000; Barak et al., 2002). Tissue distributions of PPAR α and PPAR γ gene transcripts indicate that they play roles in fatty acid catabolism (Jalouli et al., 2003; Lee et al., 2003) and adipogenesis (Chawla et al., 2003; Schiffrin et al., 2003), respectively. Much less is known about the function and regulation of PPAR δ , although it is highly expressed in the brain, colon, and skin (Braissant and Wahli, 1998; Matsuura et al., 1999; Mano et al., 2000). In the present study, concentrations of endometrial PPAR δ mRNA were greater in d-3 than in d-7 cyclic endometrium of Holstein cows. There was an inverse relationship between endometrial PPAR δ mRNA concentration and that of ER α and PGHS-2 within the first week of the estrous cycle. The physiological relevance of the inverse relationships between expression of PPAR δ and that of ER α or PGHS-2 genes in the bovine uterus is unknown and warrants further investigation. The present study would indicate that the negative correlation between PPAR δ and ER α and PGHS-2 gene expression may be specific to stage of the estrous cycle because the negative relationships no longer existed after adjustment of the mathematical models for stage of the estrous cycle. Conversely, positive Pearson and partial correlations between concentrations of ER α and PGHS-2 mRNA further support the concept that ER α may be a necessary physiological mediator of uterine PGF $_{2\alpha}$ biosynthesis in domestic ruminants.

CONCLUSION

Results provide a direct evidence for PPAR δ gene expression in the bovine uterus. Endometrial PPAR δ mRNA concentration decreased, whereas that of ER α and PGHS-2 increased between d 3 and 7 of the estrous cycle. Negative correlations between the PPAR δ gene and both ER α and PGHS-2 genes were apparently dependent on stage of the estrous cycle because the inverse relationships no longer existed when the mathematical models were adjusted for stage of the estrous cycle. Positive Pearson and partial correlations were detected between endometrial ER α and PGHS-2 mRNA concentrations, further supporting the concept that ER α may be the necessary physiological mediator of endometrial PGF $_{2\alpha}$ biosynthesis in cattle. The apparent lack of bST effect on expression of PGHS-2 was in contrast to a recent report in nonlactating Holstein cows (Guzeloglu et al., 2004), and would suggest that the net effect of supplemental bST on uterine ER α and PGHS-2 synthesis varies depending on the physiological state of the experimental animal.

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